BIOLOGY AND FINE STRUCTURE OF *CRYPTODIFFLUGIA OVIFORMIS* (RHIZOPODEA : PROTOZOA)

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Pp. 311–328; 6 *Plates*, 3 *Text-figures*

BULLETIN OF
THE BRITISH MUSEUM (NATURAL HISTORY)
ZOOLOGY Vol. 30 No. 8
LONDON: 1977

THE BULLETIN OF THE BRITISH MUSEUM (NATURAL HISTORY), *instituted in* 1949, *is issued in five series corresponding to the Scientific Departments of the Museum, and an Historical series.*

Parts will appear at irregular intervals as they become ready. Volumes will contain about three or four hundred pages, and will not necessarily be completed within one calendar year.

In 1965 *a separate supplementary series of longer papers was instituted, numbered serially for each Department.*

This paper is Vol. 30, *No.* 8, *of the Zoology series. The abbreviated titles of periodicals cited follow those of the World List of Scientific Periodicals.*

World List abbreviation:
Bull. Br. Mus. nat. Hist. (Zool.)

ISSN 0007-1498

BRITISH MUSEUM (NATURAL HISTORY)

Issued 27 January, 1977 *Price £2.75*

BIOLOGY AND FINE STRUCTURE OF *CRYPTODIFFLUGIA OVIFORMIS* (RHIZOPODEA : PROTOZOA)

By R. H. HEDLEY, C. G. OGDEN and N. J. MORDAN

CONTENTS

SYNOPSIS

Cryptodifflugia oviformis, a cosmopolitan freshwater, moss and soil inhabiting testacean has been established in clonal culture with a doubling time of between 29 and 41 hours ; it is capable of reproducing in a 14 parts per thousand saline solution. The shell is shown to have two components, a thin outer organic layer and a thick inner, non-crystalline calcareous layer. The process of reproduction by simple division and the fine structure of the vegetative stage are described in detail. Ultrastructural features of special interest are the presence of calcareous inclusions in the mitochondria ; and a storage area for polysaccharide material within the cytoplasm.

Cryptodifflugia operculata of Page (1966) is considered to be a synonym of *Cryptodifflugia oviformis* Penard (1890).

INTRODUCTION

The main characters used in the classification of testate amoebae are the form, structure and composition of the shell and the form of the pseudopodia. The shells are generally reported as being either purely organic, arenaceous, or siliceous with occasional reports of forms secreting calcareous shells being unsubstantiated. Until relatively recently information on the biology of many testate amoebae has been known only from infrequent observations on living animals, usually isolated as individuals during ecological or faunal surveys of freshwater habitats. The establishment and study of clonal cultures in this laboratory of certain species with siliceous shells (Hedley & Ogden, 1973, 1974a ; Hedley *et al.*, 1974) have contributed to our understanding of their biology and shell structure.

Recent ultrastructural studies on arenaceous forms (Netzel, 1972a; Eckert & McGee-Russell, 1974) and on organic forms (Griffin, 1972; Netzel, 1972b, 1975a, b) have contributed towards the understanding of their shell formation.

Species attributed to the genus *Cryptodifflugia* Penard, 1890, are possibly the smallest and most widely distributed of testate amoebae. There has been some doubt amongst protozoologists regarding the structure and composition of the shell in these animals with previous descriptions (Penard, 1890; Deflandre, 1953; Grospietsch, 1964; Page, 1966) reporting it as chitinous, smooth, hyaline and rigid.

The present work describes some aspects of the biology and shell structure of *Cryptodifflugia oviformis* Penard, 1890, an unusual testacean in that it is now reported as possessing a calcareous shell.

Previous work – taxonomy

The genus *Cryptodifflugia* was erected by Penard (1890) for the reception of a new species *C. oviformis*. Penard (1902) later amended his initial description and described two further species, *C. compressa* and *C. sacculus*. These three species were redescribed and illustrated by Playfair (1917) with five new species, *C. minuta*, *C. angulata*, *C. valida*, *C. crenulata* and *C. pusilla*, and four varieties, all from ponds or swamps in Australia. Zacharias (1903) reported a new species, *C. turfacea*, which sometimes had two apertures each having a distinct rim or collar. Another new species, *C. voigti*, described by Schmidt (1926) was observed to have an apertural rim and particles adhering to the shell surface.

Deflandre (1953) emended the generic diagnosis of both *Cryptodifflugia* Penard, 1890 and *Difflugiella* Cash, 1904, using the degree of compression of the shell as a main character. These emendations included the transfer of the type species, *C. oviformis*, of the genus *Cryptodifflugia* to the genus *Difflugiella*. Several authors, Bonnet & Thomas (1955, 1960), Thomas (1959), Grospietsch (1964) and Schönborn (1965), have agreed with Deflandre's changes. For example, Thomas (1959) redescribed *D. oviformis* (Penard, 1890) and tabulated the previously reported measurements of this species. In a review of the genera *Cryptodifflugia* and *Difflugiella*, Grospietsch (1964) agreed with Deflandre (1953) and gave keys to the species of both genera. Whilst Schönborn (1965) described four new species of *Difflugiella* and redescribed *D. crenulata* (Playfair, 1917), *D. oviformis* (Penard, 1890) and *D. voigti* (Schmidt, 1926).

Page (1966) reviewed the genus *Cryptodifflugia* and concluded that Deflandre's emendations were unjustified. He based his opinion on the original descriptions of *Difflugiella* and *Cryptodifflugia* which showed the former to have a flexible shell, whilst the latter had a rigid shell. Recent ultrastructural studies on *Difflugiella* sp. by Griffin (1972), and the present work, support the view that this feature is a good taxonomic character.

Page (1966) used the presence of an operculum in the aperture of encysted animals of a new species *C. operculata* to distinguish it from *C. oviformis*. This feature had not been previously described and it is difficult to determine whether its omission in earlier descriptions was due to oversight, as the operculum is often difficult to

see with the optical microscope, or to the absence of encysted animals in the material examined by other authors.

As a result of an examination of the two available cultures of *C. operculata* and *C. oviformis* (from this laboratory), Dr Page has suggested in a personal communication that the presence of an operculum may not be a good specific character, as it appears to be present in most encysted animals. Our own studies show that the structure of the shell and the fine structure of the cytoplasm in the two forms are indistinguishable. It is suggested therefore that *C. operculata* be regarded as a synonym of *C. oviformis*.

Previous work – biology

In his original description of *Cryptodifflugia oviformis*, Penard (1890) reported that the cytoplasm filled the shell and contained one nucleus, a contractile vacuole close to the nucleus and 'numerous brilliant granules'. Similar 'brilliant' or 'coarse' granules found in *C. operculata* have been shown by Page (1966) to react positively with supravital Janus green from which he concluded that they are mitochondria.

Penard (1890) observed that the form of the small number of pseudopodia of *C. oviformis* changed rapidly and later (Penard, 1902) described the pseudopodia as straight structures without ramifications. The arrangement and length of the pseudopodia in *C. oviformis* are described by Schönborn (1965), who also observed that the rate of locomotion varied from 34 to 40 μm/min. According to Page (1966) there are between two and five pseudopodia in *C. operculata* that extend from a mass of cytoplasm outside the aperture. Both this mass and the cytoplasm appear to be free from coarse granules. Although these pseudopodia appear to be filose all seem to have rounded ends. The rate of locomotion of ten individuals ranged from 14 μm/min to 28 μm/min.

United individuals were described as 'conjugating tests' by Penard (1902) in which one shell always appears empty whilst the other shell, usually the larger, contains a rounded mass of cytoplasm separated from the aperture by a 'chitinous' diaphragm. Penard suggests that this could be a resting stage during which the animal constructs a new shell that gradually hardens. Playfair (1917) has described similar united individuals of *C. oviformis* as encysted forms. The process of binary fission has been described for *C. operculata* by Page (1966), in which most of the cytoplasm to form the daughter cell is extruded before nuclear division begins and is followed by the formation of a new shell around the daughter. Nuclear division from prophase to anaphase takes place in the aboral region of the parent and the daughter nucleus moves into the extruded cytoplasm in late telophase. Both the nuclear membrane and the nucleolus disappear before metaphase and no centrioles are seen. It also appears that most of the granules pass into the extruded cytoplasm and few are left in the parent.

Penard (1890, 1902) described an encysted form of *C. oviformis* in which the cytoplasm was concentrated and contained in the aboral region behind a cyst-membrane. A similar cyst has been described by Schönborn (1965). Page (1966) reported this type of cyst only once in *C. operculata*, but regularly found cysts in which the

aperture was sealed. In these latter forms the cytoplasm filled the shell, the contractile vacuole disappears and the aperture is blocked by a lens-shaped operculum. At encystment the operculum is either rejected from the shell or ingested within the cytoplasm.

MATERIALS AND METHODS

Cryptodifflugia oviformis was isolated from a sample of moss, *Eurhynchium praelongum*, taken from the bark of a tree at Cilpost Farm, Whitland, Carmarthenshire, Wales, in July 1968. Rough cultures were obtained by allowing small portions of this sample to stand, covered by a shallow layer of culture medium, at room temperature 18–20 °C. Agnotobiotic cultures were kept in small plastic containers on a thin substrate of agar (1 per cent agar agar in distilled water) with a sterilized wheat grain added prior to setting and covered with a layer of culture medium. The medium was a 5 per cent (w/v) solution of sterilized soil extract in distilled water. Clonal cultures were established by isolating single active animals, and one such clone has been used subsequently to produce the working cultures. This clone has been deposited with, and is now maintained at, the Culture Centre of Algae and Protozoa, The Natural Environment Research Council, Cambridge, England (Reg. No. 1514/2).

Specimens of *C. operculata* obtained from the Culture Centre for Algae and Protozoa (Reg. No. 1514/1), which were initially isolated by Page (1966), have been maintained in a manner similar to *C. oviformis*. Under these conditions *C. oviformis* remains active for approximately 4 weeks, after this time mobility decreases and encysted forms predominate. Cultures may also be maintained on an agar agar substrate, streaked with the bacterium *Klebsiella pneumoniae*, but without a covering fluid. Such cultures, kept on agar slopes in closed universal bottles at 9 °C, may be sub-cultured successfully after 6 months (Dr F. C. Page, personal communication).

Cultures have been established using a graded (v/v) series of seawater to freshwater culture medium as the overlying culture fluid to the normal agar substrate. The initial experiments were made with 1, 2, 5 and 10 per cent seawater to freshwater mixtures, and in all cases active reproducing animals were observed. In subsequent experiments with higher concentrations of seawater it was found that cultures could be maintained in media up to and including 40 per cent seawater, which is the equivalent of a 14 parts per thousand saline solution.

Optical microscopy

Live animals were examined by both phase-contrast and bright-field illumination.

Scanning electron microscopy

Specimens taken from 4 or 5 weeks old cultures were cleaned by taking them individually through several changes of distilled water, using a single-hair brush.

They were allowed to dry and then transferred onto a previously cleaned cover-slip. These prepared cover-slips were mounted on a standard Stereoscan stub using an electrically conductive paint, 'Silver Dag', prior to being coated evenly with gold. The stubs were examined on a Cambridge Stereoscan MkII operating at 10 kV and the results recorded on Ilford HP4 film.

Preparations to display pseudopodia were obtained by fixing specimens *in situ* using the following method. The agar substrate in a culture vessel was wetted with a minimal quantity of culture medium, and a small number of specimens from a fresh culture were placed on this surface. After a short time the animals resumed their normal activities. The vessel was then quickly flooded with 3 per cent glutaraldehyde in cacodylic acid buffer, and left for 15 minutes. The agar surface was washed with the same buffer and selected squares were cut out and allowed to air dry. These squares were mounted on a standard Stereoscan stub using Araldite and processed as previously described.

Specimens from a culture vessel were air dried on pieces of spectographically standardized carbon rod for the analysis of inorganic chemical elements. Previous trials with this carbon rod had shown that it gave a minimal elemental background count. The pieces of rod were attached to Stereoscan stubs with Araldite. Stubs were then examined in a Cambridge Stereoscan coupled to an Ortec solid-state energy dispersive X-ray analyser, operating at 15 kV for 400 or 1000 seconds.

Electron probe analysis was carried out on specimens taken from a culture vessel and allowed to air dry onto a Stereoscan stub with a short shaft. They were coated with a thin layer of carbon and examined in a modified Cambridge Geoscan, operating at 20 kV with a specimen current of $0{\cdot}1 \times 10^{-7}$ amps.

Transmission electron microscopy

Specimens taken from cultures, approximately 2 or 3 weeks old, were fixed at room temperature for 12 minutes in 1 per cent glutaraldehyde in 0·025 M cacodylic acid buffer, followed by 7 minutes in 3 per cent glutaraldehyde in the same buffer. After several rinses in 0·1 M cacodylic acid buffer, they were post-fixed for 12 minutes in 1 per cent osmium tetroxide in distilled water. Some specimens were conventionally fixed, but before dehydration were treated with a chelating agent, 5% EDTA, for 15 minutes. The material was dehydrated, and embedded in Epon 812. Sections were cut on a Porter Blum MT2 ultramicrotome using a Du Pont diamond knife, stained in a saturated alcoholic solution of uranyl acetate and Reynold's lead citrate, and examined in an AEI 6B electron microscope operating at 60 kV. The results were recorded on Ilford EM6 plates.

The periodic acid/thiosemicarbazide/silver proteinate technique (Thiery, 1967) was carried out on thin sections mounted on gold grids. After treatment with 1 per cent periodic acid for 20 minutes at room temperature followed by several washes in distilled water, the sections were left for either 40 minutes or 18 hours in a 1 per cent solution of thiosemicarbazide in 10 per cent acetic acid. They were washed thoroughly in several changes of 10 per cent acetic acid followed by several rinses in distilled water, before treatment with 1 per cent solution of silver

proteinate for 30 minutes in the dark. The grids were washed in distilled water before examination.

Thick, unstained sections, coated with carbon, were analysed for elemental composition in an AEI EMMA-4 analytical electron microscope, equipped with a minilens, operating at 80 kV with a probe size of 0·06 μA.

DISTRIBUTION

C. oviformis is commonly found in damp and wet mosses, standing water, on aquatic plants, various soils and forest litter. A list of locality records and references was given by Bonnet & Thomas (1960). Additional localities were given by several authors, Hoogenraad & Groot (1952), Štěpánek (1963) and Bonnet (1966).

The following list shows the geographically widespread distribution :

EUROPE :	England, Wales, Scotland, Ireland, France, Belgium, Holland, Germany, Iceland, Lapland, Italy, Czechoslovakia, Hungary, Bulgaria, Russia.
NORTH AMERICA :	United States of America and Canada.
SOUTH AMERICA :	Columbia and Chile.
AFRICA :	Cameroun and the Congo.
ASIA :	Siberia.
AUSTRALASIA :	Australia and New Zealand.

SHELL STRUCTURE AND COMPOSITION

Based on measurements from a hundred specimens the ovoid, rigid and smooth shells of *C. oviformis* vary in length from 14·5 to 22·2 μm and in diameter from 12·8 to 17·6 μm. The single, terminal and circular aperture has a diameter of between 3·2 and 6·4 μm and is surrounded by a thin collar (Pl. 1, fig. A).

Ultrastructural observations show that the shell wall varies in thickness, between individuals, from 150 to 260 nm and that it is divided into two distinct layers. A thin outer organic layer, which is usually no more than 20 nm thick, and a thick inner layer of electron-dense calcified material. These layers appear to have a uniform thickness over most of the shell surface (Fig. 1), but in the apertural region there is a slight thickening of the calcified layer and an extension of the organic layer to form a circular collar (Fig. 2). This organic collar is strengthened at the internal face by a continuation of the calcified layer (Pl. 1, fig. B). Examination of the complete shell wall at the ultrastructural level is difficult because the calcified layer is not penetrated by the embedding resin, and as a result is either fractured or torn in thin sections (Pl. 2).

Whole shells, examined with an Ortec X-ray analytical attachment to the Stereoscan, showed significant elemental peaks for silicon, potassium and calcium, the last mentioned being pronounced. Semi-quantitative electron probe microanalysis of shells indicated that the calcium count-rate was high, and demonstrated a weak line for silicon corresponding to not more than 1 per cent. Sectioned material examined with the X-ray analytical electron microscope, EMMA, using a standard of calcium carbonate, gave significant intensities from the calcified layer. This

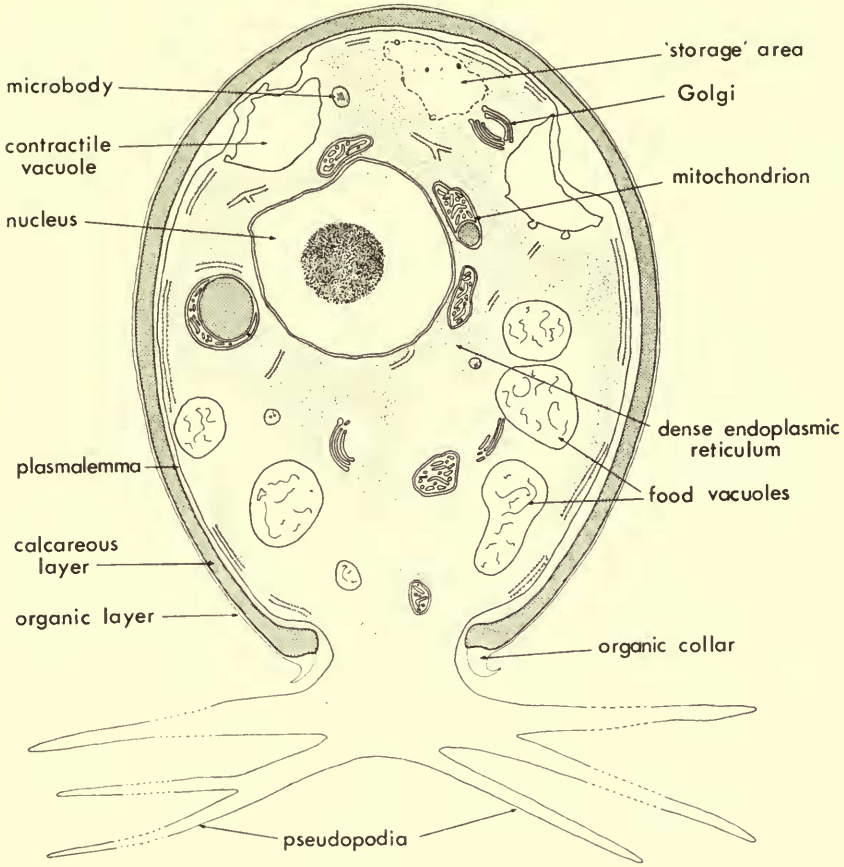

FIG. 1. Diagram of the vegetative stage of *C. oviformis* showing the arrangement of the main organelles.

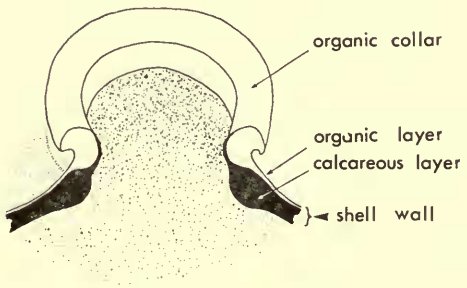

FIG. 2. Diagram of the apertural collar with a section cut away to show the relationship of the organic and calcareous layers.

layer also gave significant intensities for phosphorus, using sodium dihydrogen orthophosphate as a standard. Further attempts to analyse the composition of the calcified layer, using whole shells for X-ray diffraction and sectioned specimens for electron diffraction, were inconclusive but suggest that this material is mainly amorphous. Dr K. M. Towe (Smithsonian Institution, Washington, D.C., U.S.A.), who kindly undertook the electron diffraction analysis, suggested that the material was either amorphous or exceptionally beam-sensitive as it was unstable. He ruled out the possibility of the beam sensitivity being associated with the presence of an oxalate because there was no inversion to calcium oxide. In conclusion, we suggest that in all probability the calcified layer is amorphous calcium phosphate.

When whole specimens are immersed in either dilute nitric or hydrochloric acids or with 5% EDTA the major part of the shell dissolves. That which remains is the thin organic layer, delimiting the shape of the shell, and the organic thickening in the apertural region. In an attempt to demonstrate the fine structure of the organic layer, animals were fixed normally but before dehydration were decalcified by treatment with 5% EDTA for 10 minutes (Pl. 1, fig. C). The organic layer revealed by this method is a membrane-like structure which is thickened and folded at the aperture to form a collar (Pl. 1, fig. D). Specimens stained with the periodic acid/thiosemicarbazide/silver proteinate technique to demonstrate reactive hydroxyl groups show that the organic layer is composed of two thin, closely opposed, distinct membrane-like structures (Pl. 1, fig. E), with a high carbohydrate content.

Abnormal forms occur in cultures and represent about 3 per cent of the population. These are usually large forms, two or three times the size of a normal animal, and have two or more apertures (Pl. 1, fig. F). The shell of these animals is characterized by an uneven distribution and thickness of the outer organic covering (Pl. 1, fig. F ; Pl. 6, fig. C).

CYTOPLASM

The cytoplasm normally occupies the whole of the shell cavity (Fig. 1 ; Pl. 2) and is enclosed by a plasmalemma. The single nucleus is normally spherical, between 4·3 and 5·1 μm in diameter, and is enclosed by two tripartite membranes. The nuclear matrix is granular with small, densely stained concentrations of chromatin scattered throughout and a dense nucleolus that is usually situated centrally. The nucleus is surrounded by a densely stained mass of granular endoplasmic reticulum (Fig. 1). This region of endoplasmic reticulum appears more electron-dense than the surrounding cytoplasm due to the concentration of ribosomes. Two or three contractile vacuoles may be present in the mid-body region (Pl. 3, fig. A). The area surrounding these vacuoles is free of ribosomes but has numerous small vesicles which are often continuous with the lumen of the vacuole. The contractile vacuoles lie at the periphery of the cytoplasm and discharge directly into the shell cavity. Specimens in 2-week-old cultures discharged their contractile vacuoles at periods that ranged from 25 to 33 seconds between systole and diastole. These periods increased in animals from 6-week-old cultures

to range from 28 to 41 seconds. Numerous food vacuoles, which have a single unit membrane and may contain food organisms or waste material, occupy the apertural end of the cytoplasm (Pl. 2).

The mitochondria are ovoid or spherical, with tubular cristae, a dense granular matrix and they are distributed at random throughout the cytoplasm. Frequently large, spherical, electron-dense inclusions occur between the outer and inner mitochondrial membranes (Pl. 3, figs A and B). These inclusions are naturally electron-dense in sections from specimens fixed in glutaraldehyde but without subsequent treatment with heavy metals, and show similar fracture patterns to those sometimes seen in the electron-dense shell wall. We believe that these mitochondrial inclusions are the 'brilliant' or 'coarse' granules described by earlier workers. Analysis of these inclusions using the X-ray analytical electron microscope EMMA gave significant intensities for calcium and phosphorus which correspond to those produced by the shell. These calcareous inclusions dissolve in 5% EDTA.

As many as 6 Golgi bodies may be present in one animal and they are usually associated with concentrations of granular endoplasmic reticulum. Each dictyosome is composed of a stack of 3 to 6 saccules (Pl. 3, fig. C) which are usually concave and centrally flattened, whilst the matrix around these saccules may be noticeably less dense than the surrounding cytoplasm. Condensations of a substance, which is electron-dense with normal staining procedures, occur in the central areas of the saccules. This material appears as if it may pass to the outer margins of the saccules where they form spherical protrusions, which are budded off as smooth membrane-bound vesicles (Pl. 3, fig. C). At the inner face of the dictyosome, vesicles containing an electron-lucent material are formed (Pl. 3, fig. E). These vesicles are slightly larger than those containing electron-dense material and are lined by a thin layer of an electron-dense material (Pl. 3, fig. D). A third type of vesicle is often present in the dense granular endoplasmic reticulum (Pl. 4, fig. A). These vesicles are large, usually have electron-lucent contents, but are occasionally seen to contain an electron-dense material (Pl. 4, fig. D), and are often surrounded by a band of endoplasmic reticulum. Similar vesicles to these have been previously reported in *Trinema lineare* (Hedley & Ogden, 1974a).

On the basis of their distribution within the cytoplasm it is postulated that these three types of vesicles move through the cytoplasm along two distinct pathways. The large electron-lucent vesicles would pass through the central cytoplasm, whilst the smaller vesicles would pass around the less dense peripheral region to become incorporated at sites that are considered to be possible storage areas (Pl. 4, figs B and D). In the trophic animal there may be as many as three such storage areas, usually found on a level or posterior to the nucleus in the aboral region (Pl. 4, fig. C). These regions are electron-lucent and do not appear to have any limiting membrane.

When sections are treated with the periodic acid/thiosemicarbazide/silver proteinate technique the storage areas stain strongly, which might suggest that they are regions rich in carbodyrate. The small electron-lucent vesicles also contain the stain, but in these it is confined to the lining of the vesicles (Pl. 4, fig. B). The saccules of the Golgi apparatus are sometimes stained, as are the vesicles that are

detached at their margins, but this staining is less positive (Pl. 4, fig. B). Similar results after using this technique were obtained from specimens which had been decalcified with EDTA prior to embedding.

Microbodies are present in most of the specimens examined. They are oval or spherical, varying between 0·25 and 0·44 μm in diameter, have a dense matrix and a single unit membrane. They may contain either one or two tubular-like elements, between 19 and 24 nm in diameter (Pl. 4, fig. E), or a rectangular structure of parallel lines with a spatial distance of 8 nm between each line (Pl. 4, fig. F). Similar tubular-like elements in microbodies have been reported in the testate amoeba *Trinema lineare* (Hedley & Ogden, 1974a), and in various Foraminifera by Hedley & Wakefield (in Hruban & Rechcigl, 1969) and Febvre-Chevalier (1971). Lattice-like structures within microbodies have been previously described in the Foraminifera *Shepheardella taeniformis* and *Globigerina bulloides* by Hedley *et al.* (1967) and Febvre-Chevalier (1971).

PSEUDOPODIA

In recent classifications (Loeblich & Tappan, 1961 ; Honigberg *et al.*, 1964) the class Rhizopodea has been divided – using the form of the pseudopodia as the distinguishing character – into three subclasses ; the Lobosia, Filosia and Granuloreticulosa. In both classifications the superfamily Cryptodifflugiacea is included in the sub-class Lobosia. Previous studies (Penard, 1902 ; Page, 1966) demonstrate that the pseudopodia of *C. oviformis* appear to be intermediate in form between lobose and filose structures. Recognizing this difficulty, Deflandre (1953) erected a new sub-order the Reticulolobosa, but this has not been adopted in subsequent classifications.

In actively moving specimens of *C. oviformis* the pseudopodia are numerous, relatively long and appear to taper and have small branches. All these features are normally associated with filose pseudopodia (Pl. 5, fig. A).

Micrographs of whole animals which had been found with the pseudopodia extended (Pl. 5, fig. C) indicate that in addition to the main filose pseudopodia as seen with optical microscopy there are numerous ultrastructural pseudopodia or pseudopodial strands. These may or may not be connected to form a reticulum. These ultrastructural pseudopodia are similar to those seen in the granuloreticulose pseudopodia described for various foraminiferans by Hedley *et al.* (1967), Marszalek (1969) and Lengsfeld (1969).

Each pseudopodium appears to consist of ground-plasm, limited by a unit membrane, often containing bands of thick microfilaments and small electron-dense concentrations (Pl. 5, fig. B). In addition, some sections of pseudopodia contain strands of endoplasmic reticulum, Golgi bodies, membrane-bound vesicles and mitochondria, some of which may contain electron-dense calcium inclusions. The bands of thick microfilaments vary from 25 to 33 nm in diameter and are about 0·4 μm long (Pl. 5, fig. B). Microfilaments are sometimes found concentrated to form adhesion plaques at points of contact between opposing pseudopodia (Pl. 5, fig. D). We suggest that this reaction is probably restricted to contact between

pseudopodia from different animals. Similar adhesion plaques have been reported in species of *Euglypha* by Hedley & Ogden (1973, 1974b).

The fine structure of the pseudopodia of *C. oviformis* is similar to that reported for other testate amoebae (Wohlman & Allen, 1968; Griffin, 1972; Eckert & McGee-Russell, 1973; Hedley & Ogden, 1973, 1974a) and naked amoebae (Comly, 1973; Taylor *et al.*, 1973; Rinaldi & Hrebenda, 1975) in having microfilaments as a structural element. Wohlman & Allen (1968) and Eckert & McGee-Russell (1973) suggest that both thin and thick microfilaments are associated with pseudopodia extension and contraction in *Difflugia*, and similar studies on slime moulds and naked amoebae by Komnick *et al.* (1972, 1973) suggest that the uptake and release of calcium ions by microfilaments plays an important role in cytoplasmic streaming. Another role for both types of microfilaments, that of cellular wound healing, has recently been suggested by Jeon & Jeon (1975) from studies on *Amoeba proteus*.

REPRODUCTION

The length of time required to double the population (doubling time) was estimated from three identical cultures established and maintained under similar conditions. Growth curves produced from observations made at regular intervals showed that the doubling time is between 29 and 41 hours.

The formation of a daughter cell by binary fission begins with an elongation of the cytoplasm of the parent shell to form a thick trunk which emerges from the aperture (Fig. 3·1). This trunk expands until the cytoplasm has reached about half the size of the parent shell. There is then a slight contraction, followed by further expansion until it reaches a size equivalent to that of the parent shell. At about this time the first indication of the transfer of cytoplasmic organelles becomes apparent, with the rapid passage of the dense granules into the daughter. The parent cytoplasm contracts slightly and is less active for a period of approximately 1 minute. The daughter then collapses slightly and the granules return to the parent. The rapid movement of the granules between the parent and daughter is repeated, and finally most of the granules are passed into the daughter with only one or two remaining in the parent (Fig. 3·6). The cytoplasm in the parent is reduced, to about one-third its normal volume and is situated at the aboral region of the shell, whilst the daughter cell appears to have reached full size. The connection between the two animals is reduced to a thin thread (Fig. 3·8). As the parental cytoplasm moves towards the apertural region of the shell, the connection between the parent and daughter is severed. Pseudopodia emerge from the parent and the two animals separate, the daughter remaining quiescent for periods in excess of 45 minutes from the time of separation.

The process of granular exchange takes place between 15 and 25 minutes after the first stages of division. Contractile vacuoles appear in the daughter after about 20 minutes, and the nuclei appear in both animals after about 30–35 minutes.

Often animals appear to start dividing only to cease at various stages, including stages that are almost half the parental size. These abortive products are rejected

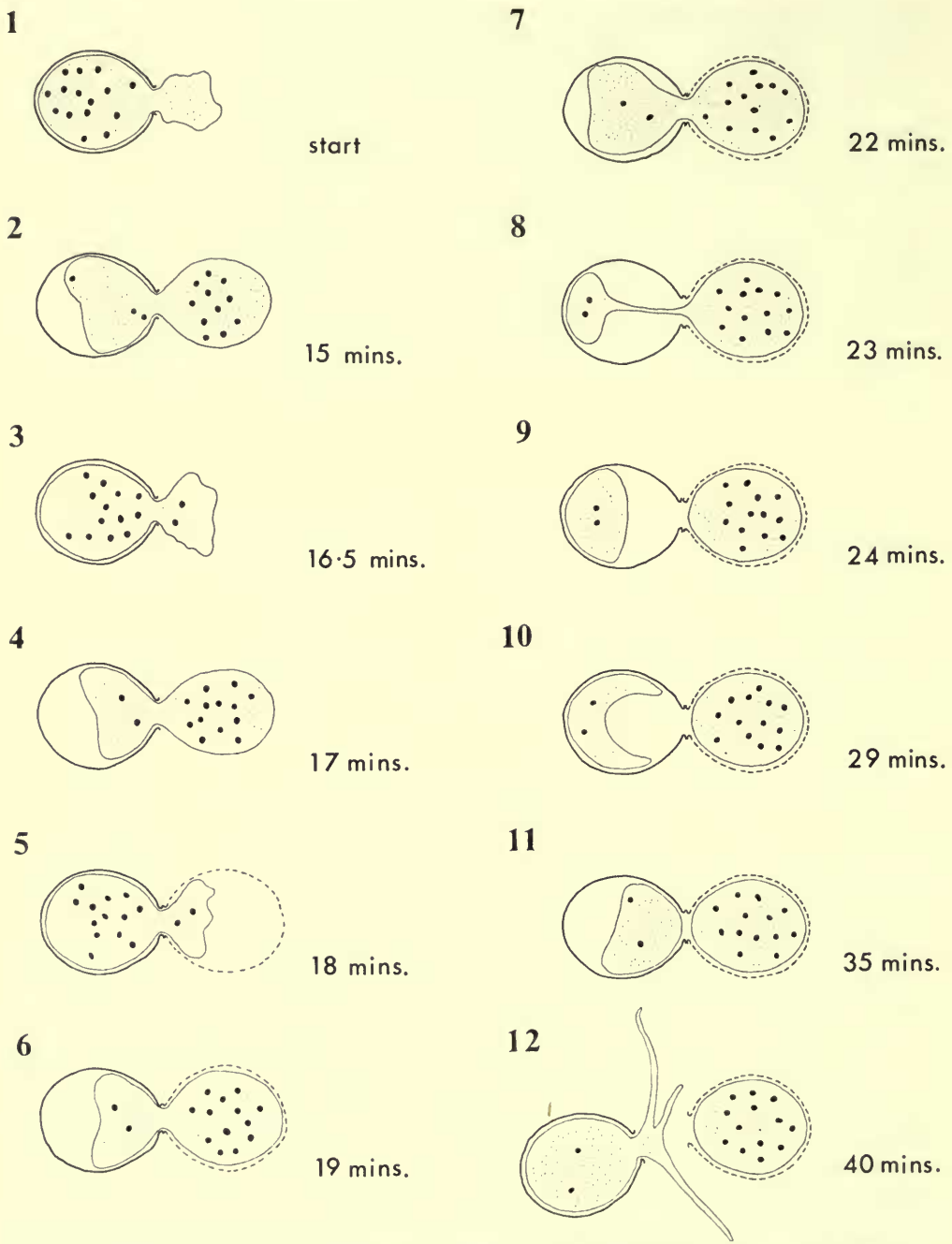

FIG. 3. Diagram showing the progressive stages in the formation of a daughter cell. At stage 1 the animal appears to rest for up to 15 minutes. Stages 2–7 represent the cytoplasmic contractions and rapid interchange of dense granules between the parent and daughter. Stage 5 shows the first indication of a thin shell (pecked line) surrounding the daughter, although this is probably present at an earlier stage. By stage 9 the cytoplasm has been divided between the parent and daughter and at stage 12 the parent moves away.

by the parent to become isolated, rounded lumps of cytoplasm, which are often seen in the cultures.

The first indication of a shell surrounding the newly formed daughter cell is a thin organic layer, indications of which are first seen after the start of granular exchange between parent and daughter. Nevertheless, it is possible that this organic layer is present around the initial extrusion of cytoplasm at the beginning of division. The formation of the shell in *C. oviformis* is probably dependent on the accumulation within the parent of reservoirs of an acid mucopolysaccharide and calcium ions. The organic material we believe is produced by both the Golgi complexes and the dense endoplasmic reticulum, and is stored in the cytoplasm at the storage areas (Pl. 3, fig. A ; Pl. 4, fig. B). The fusion of vesicles to form storage areas appears to represent a process of intake and assimilation. There is no evidence to suggest how this material is distributed from this reservoir, but the storage areas are absent in young daughter cells. A similar aggregation of vesicles rich in polysaccharides has been demonstrated by Holtrop (1972) in cartilage cells of the epiphyseal plate, where the protein polysaccharide complex was considered to assemble before it was extruded into the matrix.

Calcification of the shell appears to start shortly after division. During the early stages of calcification the shells often have an irregular outline (Pl. 6, fig. B), and they appear to be flexible. This initial shell wall has a diffuse appearance, with some small concentrations of electron-dense material on the inner surface (Pl. 5, fig. E). When the parent and daughter cells have finally divided they separate and the aperture of the daughter is sealed by a thin, fibrous organic membrane (Pl. 6, fig. A). In the cytoplasm of the daughter cell the usual membrane-limited profiles of endoplasmic reticulum are absent and it appears to be a mass of free ribosomes. The small circles of ribosomes that are seen (Pl. 5, fig. E) are possibly the initial stages in the reformation of these profiles.

In *C. oviformis* it has been possible to locate only one site where calcium is concentrated. This is between the outer and inner mitochondrial membranes where it is stored as spherical inclusions of amorphous calcium phosphate (Pl. 3, fig. B). Most of these mitochondria pass at division into the daughter cell. Several young animals examined shortly after division contain mitochondria without inclusions and in such cases the matrix of the mitochondria appears to be homogeneous, the cristae are not readily resolved, and the outer mitochondrial membrane is seen to have small swellings (Pl. 5, fig. F). It has been established by Wasserman & Kallfelz (1970) that calcium ions are actively transported across mitochondrial membranes, and a model for this transport has been proposed by Rasmussen (1966). Previous investigations (Greenawalt *et al.*, 1964 ; Greenawalt & Carafoli, 1966 ; Thomas & Greenawalt, 1968) on calcium-rich granules in mitochondria suggest that formation takes place in three stages. First at the inner membrane surfaces, especially on the cristae, followed by free granules in the matrix and finally the granules grow by accumulation.

Opinions differ over the factors that induce concentrations of calcium in the mitochondria of the ciliated protozoan *Spirostomum ambiguum*, where they have been considered to be a mobile endoskeleton (Bien, 1967), a metabolic store of

phosphate (Pautard, 1970), or concentrations of waste calcium (Jones, 1967, 1969). But in vertebrates it has been suggested that similar mitochondrial inclusions to those demonstrated here in *C. oviformis* are involved in mineralization of deer antlers (Sayegh *et al.*, 1974) and eggshell formation in chickens (Hohman & Schraer, 1966).

CYST

After 4 or 5 weeks encysted animals appear in cultures. Such animals have a distinct operculum forming a seal across the aperture (Pl. 6, fig. E) and the ultrastructure of this operculum is a finely fibrillar matrix interspersed with small naturally electron-dense bodies (Pl. 6, fig. D). Results from periodic acid-Schiff tests carried out by Page (1966) suggest that the operculum is mainly of a polysaccharide nature.

ACKNOWLEDGEMENTS

We would like to thank Dr F. Page of the Culture Centre for Algae and Protozoa, NERC, Cambridge, for his helpful discussions on the identification of *C. oviformis*, Dr K. M. Towe (Smithsonian Institution, Washington, D.C., U.S.A.) who offered observations after electron diffraction examination of the shell, and our colleague Dr C. R. Curds who kindly read and discussed the typescript.

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PLATE 1

Cryptodifflugia oviformis

FIG. A. Specimen to illustrate the circular aperture and the apertural collar (arrowed). × 2500

FIG. B. Section showing one side of the apertural collar. Note that the calcareous layer continues as a border (arrow) to the organic collar (oc). × 30 100

FIG. C. Section through the aperture of a specimen treated with EDTA to remove the calcareous layer. Note the thin organic layer (ol) and thickened collar (oc). × 5800

FIG. D. Section through aperture of specimen treated with EDTA, to show the extension of the organic layer (ol) to form the collar. × 27 000

FIG. E. Section of shell wall stained with thiosemicarbazide/silver proteinate, showing silver deposited at the organic layer (ol). The gap (g) represents the calcareous layer removed during the staining technique. × 24 000

FIG. F. Abnormal shell showing irregular patterning of the shell and two apertural openings (arrowed). × 1600

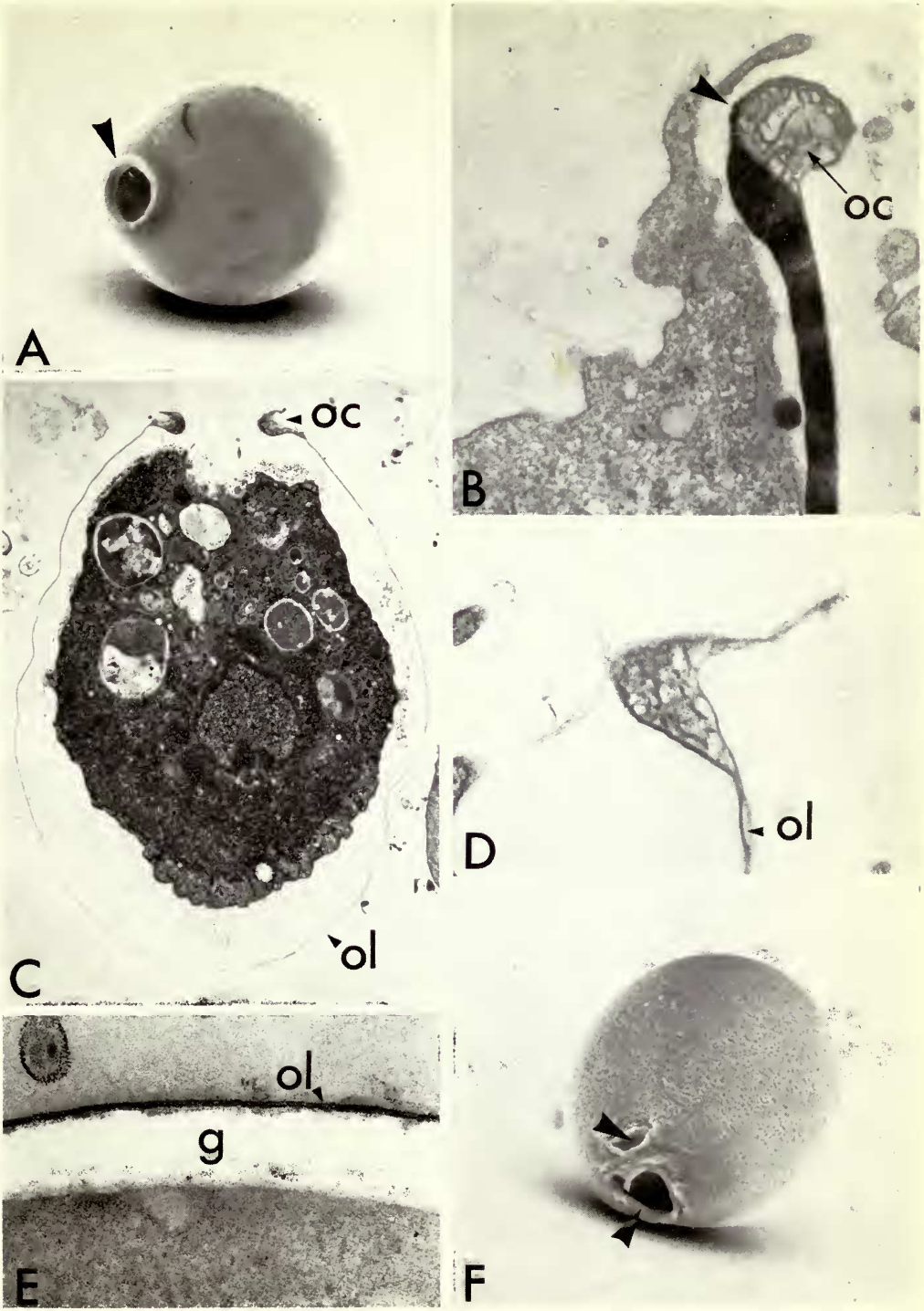

A
B
oc
C
oc
ol
D
ol
E
ol
g
F

PLATE 2

Cryptodifflugia oviformis

Section through the aperture of a whole specimen to show the nucleus (n), Golgi bodies (G), granular endoplasmic reticulum (ger) and food vacuoles (fv). ×8600

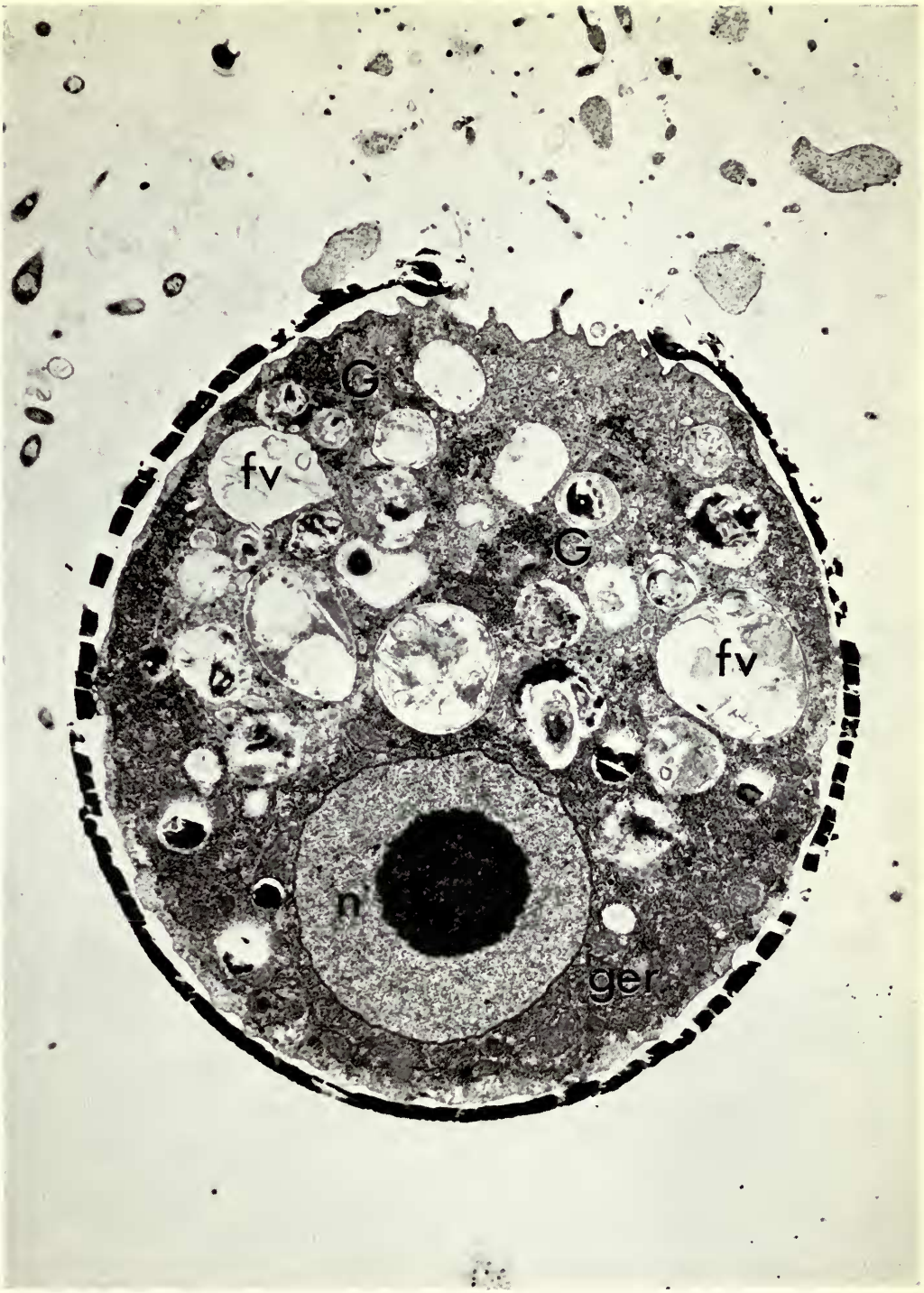

G
fv
G
fv
n
ger

PLATE 3

Cryptodifflugia oviformis

FIG. A. Section to show the nucleus (n), contractile vacuoles (cv), storage area (sa) and mitochondria containing calcareous inclusions (mc). × 7800

FIG. B. Section of a mitochondrion (m) with a calcareous inclusion. × 15 600

FIG. C. Section through a Golgi body to illustrate the formation of vesicles with electron-dense contents at the margin of the saccules (arrowed). × 44 600

FIG. D. Section showing membrane-bound vesicles (mbv) with a thin electron-dense lining and an electron-lucent centre. × 44 600

FIG. E. Two Golgi bodies (G) in a region of dense endoplasmic reticulum. Note the presence of membrane-bound vesicles (mbv) at the inner face of the saccules. × 30 100

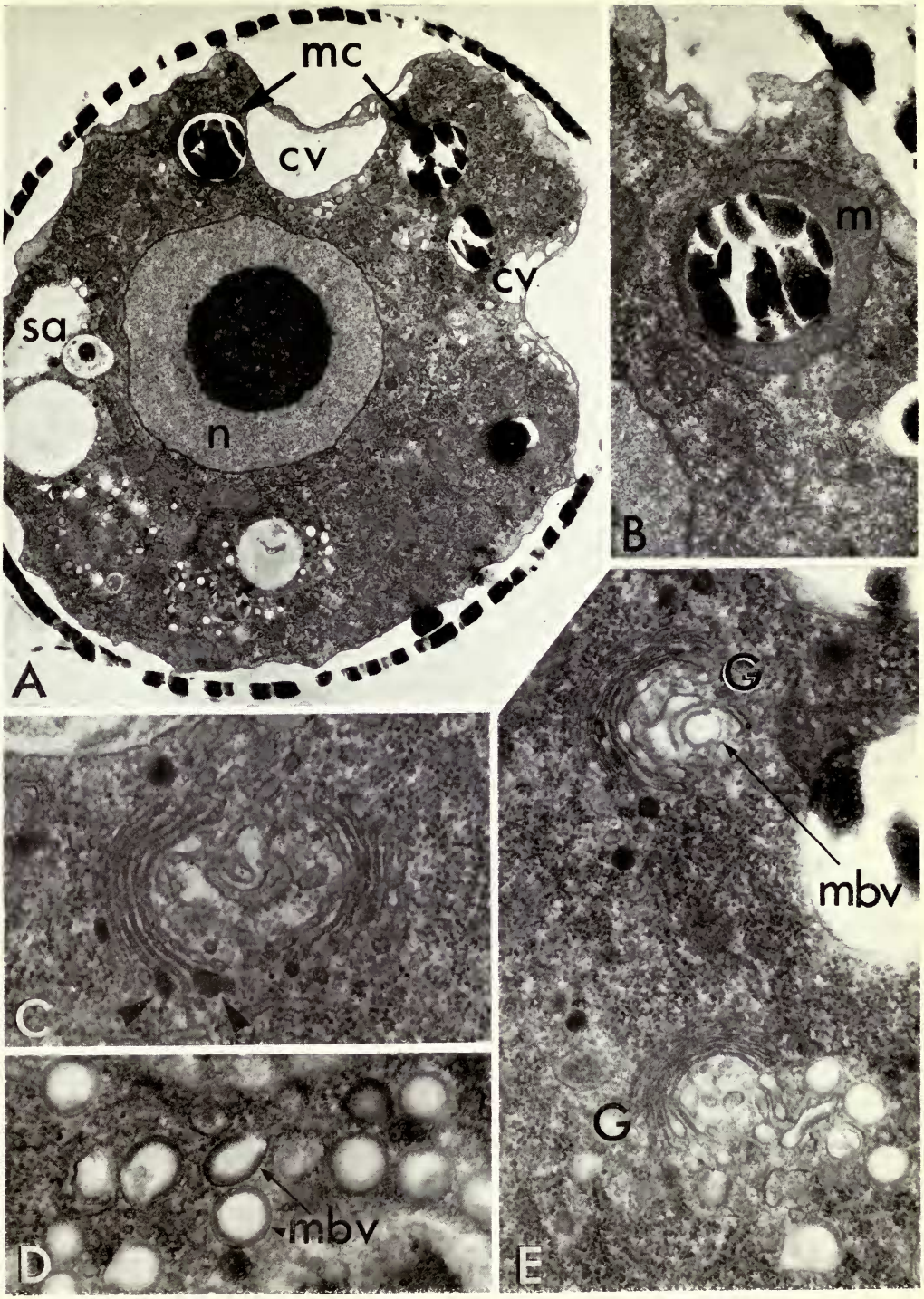

mc
cv
cv
sa
n
A
m
B
G
mbv
G
E
C
mbv
D

PLATE 4

Cryptodifflugia oviformis

FIG. A. Section to illustrate a large electron-lucent vesicle (lv) and the associated band of endoplasmic reticulum (er). × 11 500

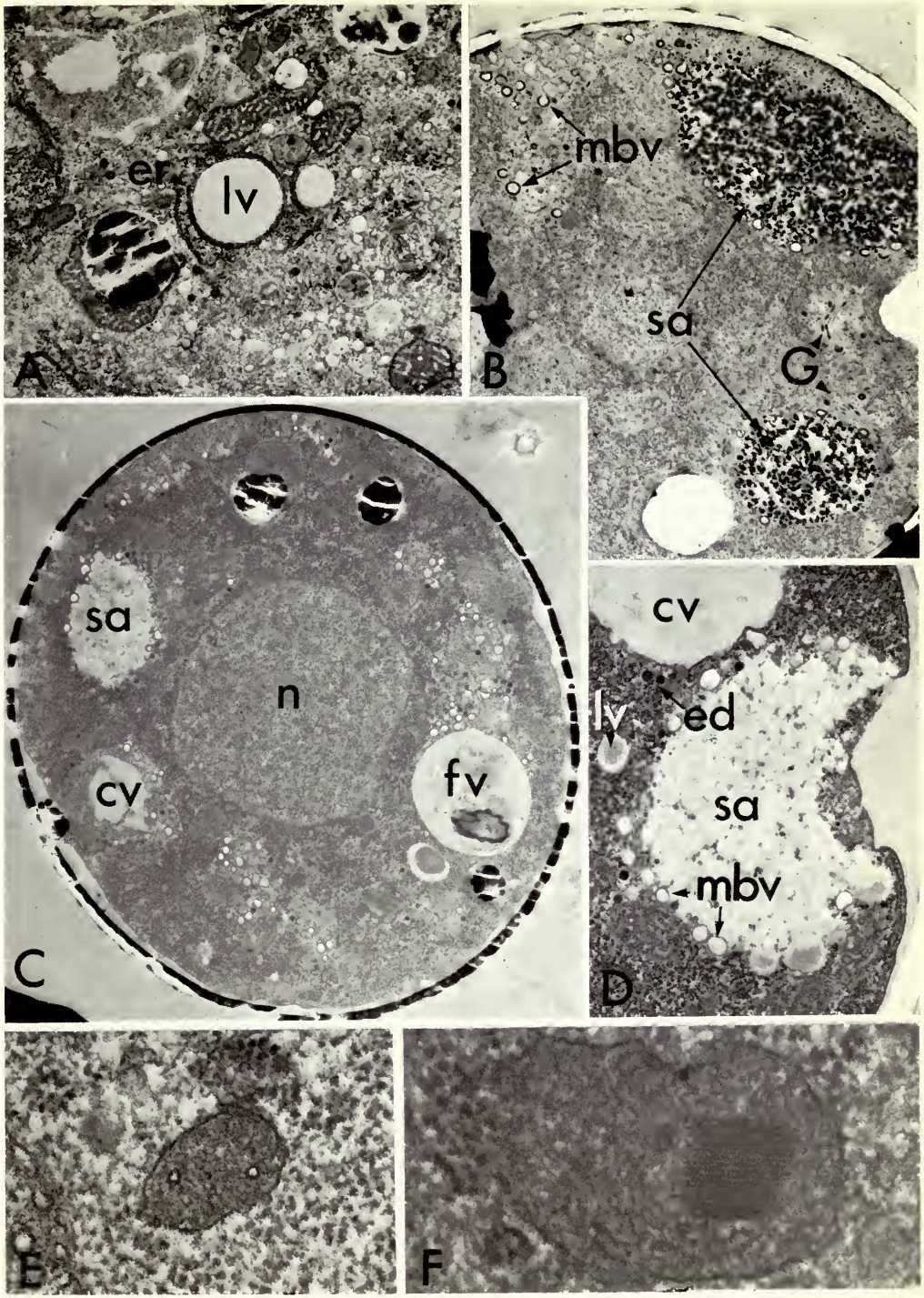
FIG. B. Section stained with thiosemicarbazide/silver proteinate, showing silver deposited at the storage areas (sa), Golgi bodies (G) and the membrane-bound vesicles (mbv). × 10 000

FIG. C. Transverse section showing the position of the storage areas (sa), food vacuole (fv), contractile vacuoles (cv) and nucleus (n). × 7800

FIG. D. A typical storage area showing three types of vesicles, electron-dense (ed), membrane-bound (mbv) and large vesicles (lv). Note that the large vesicles contain an electron-dense material. × 13 500

FIG. E. Section through a microbody with tubular elements. × 75 600

FIG. F. Section through a microbody containing a lattice-like structure. × 90 600


er
lv
A
mbv
sa
G
B
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n
cv
fv
C
cv
lv
ed
sa
mbv
D
E
F

PLATE 5

Cryptodifflugia oviformis

FIG. A. Specimen on an agar surface to illustrate the pseudopodia extended. ×960

FIG. B. Section through a pseudopodium containing mitochondria (m), bundles of microfilaments (mf) and small electron-dense concentrations (arrowed). ×44 600

FIG. C. Section of a specimen to illustrate the range and abundance of pseudopodia. ×4300

FIG. D. Section through two pseudopodia showing that the opposed membranes are parallel in the region of contact. Note the thickening of those membranes (arrowed) and the bundles of microfilaments (mf). ×15 000

FIG. E. Section of partially calcified daughter cell, showing the organic layer (ol) with small concentrations of electron-dense material at the inner surface. ×20 100

FIG. F. Section of a mitochondrian with a homogeneous matrix and apparent absence of cristae. A swelling of the membrane is arrowed. ×30 100

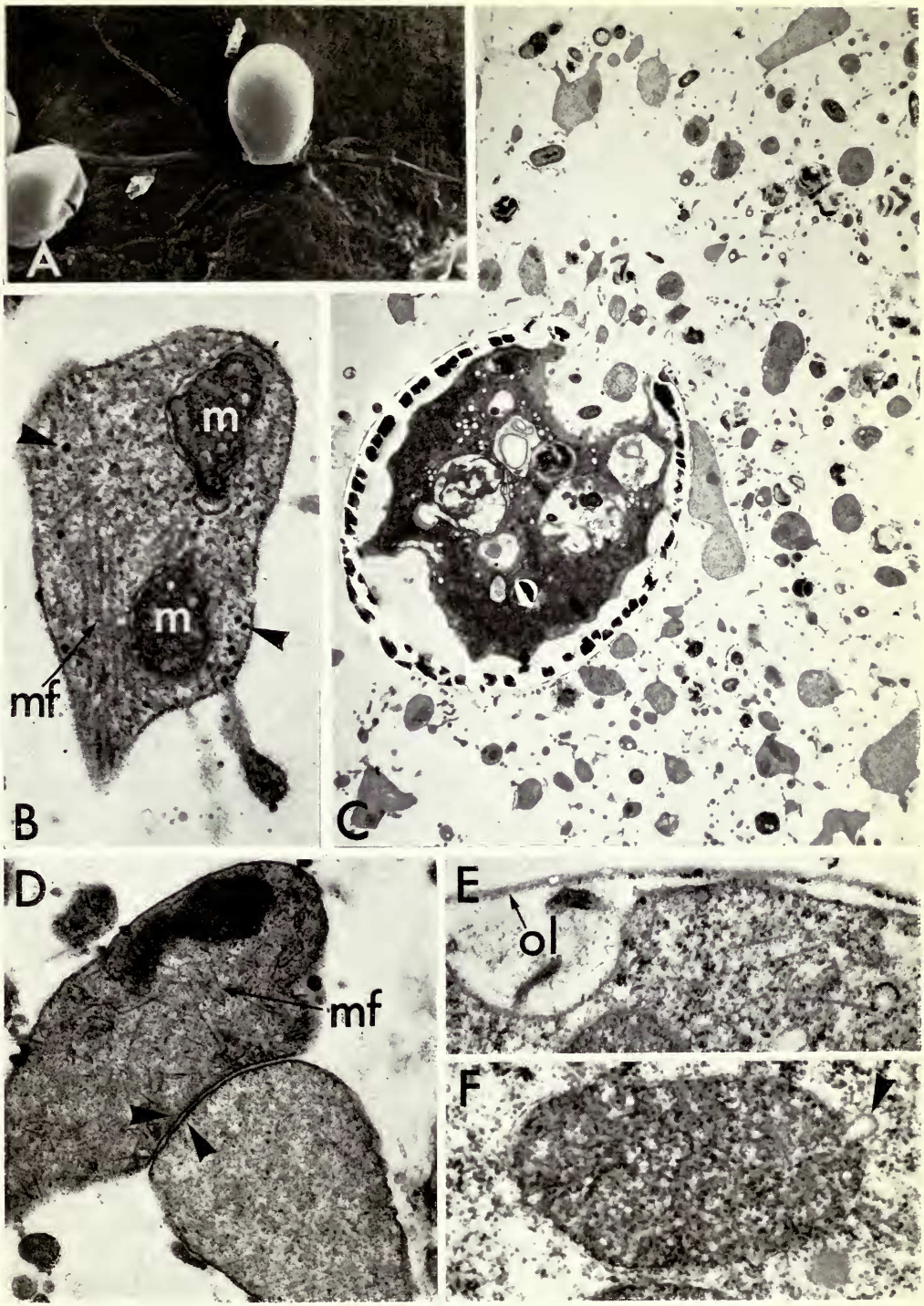

A
B
m
m
mf
C
D
mf
E
ol
F

PLATE 6

Cryptodifflugia oviformis

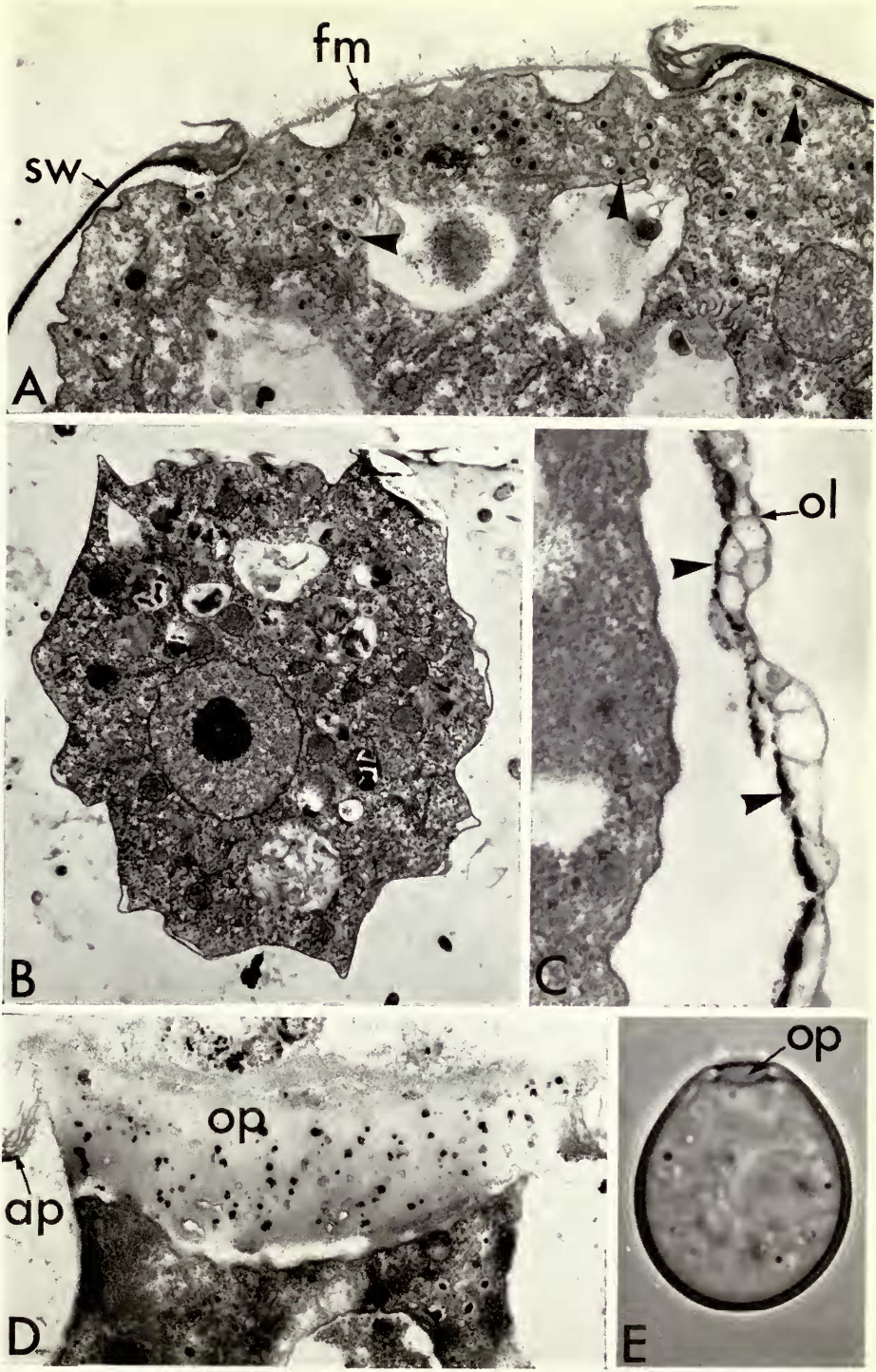
FIG. A. Section through the aperture of a partially calcified daughter cell. Note the thin shell wall (sw), the band of fibrillar organic material (fm) sealing the aperture and the vesicles containing electron-dense material (arrowed). × 20 100

FIG. B. Section of a young daughter cell illustrating the irregular outline of the uncalcified shell. × 5800

FIG. C. Section of shell wall of abnormal specimen. Note the folded organic layer (ol) and the small concentrations of calcified material on the inner surface (arrowed). × 30 100

FIG. D. Section through an aperture (ap) sealed with an operculum (op). × 28 400

FIG. E. Light micrograph showing the operculum (op) in the apertural opening. × 2000


fm
sw
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C
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ap
D
op
E